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NAVICULAR DISEASE: BIOCHEMICAL STUDIES OF SYNOVIAL FLUIDS AND TISSUES INVOLVED.

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Academic Dissertation

To be presented with the permission of Faculty of Veterinary Medicine for public examination in Auditorium Maximum, Hämeentie 57, 00580 Helsinki, on August 22nd , 2003 at 12 noon.

Helsinki 2003

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ISBN 952-10-1297-8 (PDF version)
ISBN 952-91-6138-7 (paperback)

CONTENTS

1. ACKNOWLEDGEMENTS

2. LIST OF ORIGINAL PUBLICATIONS

3. ABBREVIATIONS

4. ABSTRACT

5. INTRODUCTION

6. REVIEW OF LITERATURE

6.1 Anatomy of the navicular area

6.2 Communication between the navicular bursa and the distal interphalangeal joint

6.3 Analysis of synovial fluid and the navicular bursal fluid

6.4 Analysis of the cartilage and the tendon matrix

6.5 Navicular disease

6.5.1 Aetiology

6.5.2 Clinical signs and diagnosis

6.5.3 Treatment of the navicular disease

6.6 Influences of loading

6.7 Joint pressure

6.8 Imbalance of the hoof and corrective shoeing

7. AIMS OF THE STUDY

8. MATERIALS AND METHODS

8.1 Horses

8.2 Handling of samples

8.2.1 Synovial and intrabursal fluid (II, III)

8.2. 2 Cartilage and tendon samples (IV)

8.3 Procedures

8.3.1 Injection techniques

8.3.2 Radiography

8.3.3 Papain digestion

8.3.4 Tissue extraction
8.3.5 Determination of DNA
8.3.6 Cartilage oligomeric matrix protein concentration
8.3.7 Glycosaminoglycan concentration
8.3.8 Hyaluronan concentration
8.3.9 Determination of MMPs
8.3.10 Total protein concentration
8.3.11 Loading apparatus
8.3.12 Measuring intra-articular pressure
8.3.12 Silicone casting
8.4 Statistical analysis

9. RESULTS

9.1 Injection techniques (I, II, III, IV)
9.2 Different control tissues (II, III, IV)
9.3 Navicular hyaline and fibrocartilage and deep digital flexor tendon from horses with navicular disease (III, IV)
9.4 Pressure and contact area (V)

10. DISCUSSION
11. CONCLUSIONS
12. REFERENCES

1. ACKNOWLEDGEMENTS

This work was carried out at the Department of Farm Animal and Equine Medicine, Royal Veterinary College, and in the Faculty of Veterinary Medicine, University of Helsinki. While there are obviously many people who I cannot thank by name, I would like to express my sincere thanks to the following people who have contributed to the work presented here, and without whom I would not have been able to complete this thesis.

My supervisors, Professors Stephen May and Riitta-Mari Tulamo. It has been an honour to work under Professor May's guidance. His vast knowledge base and endless energy continually managed to astound me. Riitta-Mari presented the initial idea of the study to me and introduced me to the world of science. Without her encouragement this thesis would still be unfinished.

Dr Joe Bird for passing on some of his expertise in paper writing and laboratory techniques, for giving me an idiots guide to biochemistry and for always being available to talk.

Rosemary Forster for invaluable and cheerful help.

Dr Alan Wilson for his generous guidance and his optimistic attitude that everything would eventually work out just fine.

Drs Päivi Maisi and Saara Raulo, they introduced me to zymography.

Dr Michael Schramme for teaching me the secrets of injecting the navicular bursa and for valuable criticism and useful discussions.

Dr Roger Smith for his enthusiastic attitude. He has the gift of seeing something interesting in the most mundane results.

Dr Olli Makela for collecting the synovial fluid samples.

Dr Martin Weaver for getting me started with this research and for cheerful help.

Dr Michael Bayliss for constructive criticism and good advice.

Dr Polly McGuigan, Kate Rogers, Dr Helen Birch, Sylvia Reedy, Dr Tracey Smith, Linda Williams, Dr Satu Sankari and Merja Ranta for helping me in the laboratory. I wasn't the most talented lab student, but they managed to get me through the assays.

Dr Julie Martin, Dr Javed Igbald, Dr Rodolfo Cappello, Dr Eddy Cauvin and Dr Shelley Goodman for scientific advise and for being able to make me laugh, in the most adverse of circumstances.

My parents, Eeva and Juhani Kuusio,for providing me with a healthy foundation for life.

Jaakko, Saara, Elsa and Ida for giving me so much joy and happiness.

Mika, my dear husband, for supporting me in every way over the last years.

The Financial support from the Finnish Veterinary Foundation, the Erkki Rajakoski Foundation, the Finnish Veterinary Practitioners, the Farmos Research and Science Foundation, the Home of Rest for Horses, the Royal Veterinary College, London, and the Faculty of Veterinary Medicine, Helsinki, is gratefully acknowledged.

2. LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

I Schramme M, Boswell J, Hamhougias K, Toulson K, Viitanen M. An in vitro study to compare 5 different techniques for injection of the navicular bursa in the horse. Equine Veterinary Journal 32: 263-267, 2000.

II Viitanen M, Bird J, Maisi P, Smith R, Tulamo R-M, May S. Differences in the concentration of various synovial fluid constituents between the distal interphalangeal joint, the metacarpophalangeal joint and the navicular bursa in normal horses. Research in Veterinary Science 69: 63-67, 2000.

III Viitanen M, Bird J, Mäkelä O, Schramme M, Smith R, Tulamo R-M, May S. Synovial fluid studies in navicular disease. Research in Veterinary Science 71:201-206, 2001.

IV Viitanen M, Bird J, Smith R, Tulamo R-M, May S. Biochemical characterisation of navicular hyaline cartilage, navicular fibrocartilage and the deep digital flexor tendon in horses suffering from navicular disease. Research in Veterinary Science (in print).

V Viitanen M, Wilson A, McGuigan P, Rogers K, May S. The effect of foot balance on the intra-articular pressure in the distal interphalangeal joint *in vitro*. Equine Veterinary Journal 35:184-189, 2003.

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3. ABBREVIATIONS

COMP	cartilage oligomeric matrix protein
DDFT	deep digital flexor tendon
DIP	distal interphalangeal
DSIL	distal sesamoidean impar ligament
ECM	extracellular matrix
GAG	glycosaminoglycan
GnHCl	guanidine hydrochloride
HA	hyaluronan
HU	heel up
LC	cartilage of the foot
LU	lateral side up
M	meniscus
MCP	metacarpophalangeal joint
MMP-2	matrix metalloproteinase 2
MMP-9	matrix metalloproteinase 9
MRI	magnetic resonance imaging
MU	medial side up
NA	navicular hyaline cartilage
NB	navicular bursa
NF	navicular fibrocartilage
NSAIDs	non-steroidal anti-inflammatory drugs
PDN	palmar digital nerve block
PGE_2	prostaglandin E_2
PSGAGs	polysulphated glycosaminoglycans
OA	osteoarthritis
SF	synovial fluid
T	tendon
TU	toe up

4. ABSTRACT

Markers for navicular disease, particularly molecules associated with the mechanisms involved in fibrocartilage degeneration, were investigated. Synovial fluid has been used as an indicator of articular pathology for decades. The preliminary study was undertaken to establish a reliable injection technique into the navicular bursa (NB) to obtain navicular bursal fluid for biochemical analyses. A further objective was to obtain reference values for constituents of normal navicular bursal fluid and to compare these values with those obtained from the normal distal interphalangeal and metacarpophalangeal joints. We hypothesized that if communication, or filtration exists, between the bursa and the distal interphalangeal joint (DIP joint), then synovial fluids (SF) collected from these cavities, from the same horses, should be very similar.

Synovial fluids from the NB and DIP joint were also analysed from horses suffering from navicular disease and compared with the healthy control group. Synovial fluid was analysed for presence of the major cartilage macromolecules. The disturbance of metabolic turnover, as is found in osteoarthritis (OA), may be reflected in changes in the profile of biochemical degradation products in the SF.

Our aim was to identify the same biochemical parameters from the tissues involved in navicular disease as those measured from SF. To this end, we wished to establish whether the biochemical events involved in the turnover of tissues, were reflected in the composition of the SF. Samples were collected from the deep digital flexor tendon (DDFT) and navicular fibrocartilage (NF). We also wanted to investigate the involvement of the DIP joint in navicular disease; thus, analyses were performed on

synovial fluids from the DIP joint, and navicular hyaline cartilage (NA) was also analysed in diseased horses.

In addition, the biochemistry of navicular fibrocartilage was compared with other fibrocartilages since fibrocartilage composition is thought to vary according to anatomical location, and consequently, may respond differently to disease processes. Fibrocartilage was collected from the cartilages of the foot and from the meniscus of the femorotibial joint.

One of the most common treatments for navicular disease is corrective shoeing. However, little is known about how shoe or foot alteration affects tissues in adjacent regions. Egg-bar shoes or heel wedges are used in an attempt to correct the hoof balance. Horses with navicular disease are thought to alter their gait in response to pain in the heel region. Alterations in the joint angle may increase the intra-articular pressure of the DIP joint and thus cause pain. The intra-articular pressure of the DIP joint and joint volume changes were therefore measured in cadaver limbs loaded in a balanced position and in limbs positioned with heel up, medial side up, lateral side up and toe up. We aimed to show how changes in one region of the hoof might simultaneously affect other areas.

Synovial fluids from horses with normal metacarpophalangeal (MCP joint) and DIP joints, and NB demonstrated a significant correlation between molecular parameters in the DIP joint and NB, but no correlation with the fluids measured in the MCP joint. Reduction in glycosaminoglycan (GAG) concentration in both the DIP joint and NB of horses with navicular disease was noted, as well as an increase in hyaluronan concentration (HA) in the DIP joint, and an increase in metalloproteinases MMP-2 and

MMP-9 in the DIP joint and NB. However, the correlation of the concentrations of the different molecules measured between the DIP joint and NB seen in healthy horses was not maintained in disease, suggesting that the changes from normal condition, which were recognized in the end synovial compartment, were a reflection of parallel disease processes rather than molecular equilibration between adjacent synovial structures. Moreover, biochemical changes have been demonstrated to occur in tissues other than fibrocartilage from diseased joints. Horses with navicular disease had increased concentrations of cartilage oligomeric matrix protein (COMP) in their navicular hyaline cartilage. MMP-2 levels were increased in the DDFT and navicular hyaline cartilage, while GAG content decreased in navicular hyaline and fibrocartilage in diseased joints.

The in vitro loading study showed that changes in hoof angle resulted in a redistribution of load over the DIP joint and alterations in the intra-articular pressure within the joint. Any alteration from the "ideal" foot balance decreased the DIP joint contact area, thus increasing the load per unit area in comparison with the even contact across the articular cartilage of the distal phalanx and the navicular bone in a balanced foot.

5. INTRODUCTION

Navicular disease is a condition of major economic importance in the horse, and has considerable welfare implications, accounting for 33% of all chronic foreleg lamenesses (Colles 1982). A broad definition of navicular disease is that it is a chronic, usually bilateral forelimb lameness that fits a specific set of diagnostic criteria (Stashak 2002). Navicular disease (or syndrome because exact cause is not known) involves at least one of several structures in the palmar aspect of the foot, including the navicular bone, DIP joint, navicular bursa, DDFT, and collateral ligaments of the navicular bone and distal sesamoidean impar ligaments. Ideally, we should restrict the term navicular disease only to horses with known pathological abnormalities of bone but with current diagnostic methods available to most practitioners it is not always possible to do so. Probably horses with no radiological changes have primary soft tissue injuries; there is a need to find out different causes of palmar foot pain in horses. While reports of navicular disease date back about 200 years (Weaver 2001), the aetiology, pathogenesis and lesions responsible for lameness remain controversial. Some of this controversy and frustration arises because of the difficulty in specifically diagnosing the source of heel pain in a given horse. In the last 10-15 years, evidence has accumulated to support the hypothesis that navicular disease is a degenerative condition of the bone; 'a wear-and-tear' phenomenon analogous to OA.

The diagnosis of navicular disease is usually based on history, clinical signs, response to analgesia of the palmar digital nerves and detection of radiographic abnormalities (Turner 1989). However, clinical signs of navicular disease, without any radiological

abnormalities have been described (Verschooten et al. 1989). Today, we have still only developed criteria to predict the tissue destruction and progression of navicular disease, or OA, in a relatively late stage of the disease. It would be an advantage to have biochemical markers of early disease activity and joint destruction, to optimize therapy.

Synovitis and capsulitis due to repeated trauma are common initial changes in the joints of athletic horses (McIlwraith and vanSickle 1981). Injury or insult to the synovial membrane results in the release of degradative enzymes, particularly neutral metalloproteinases, which degrade cartilage matrix (Caron 1992, Arnoldi et al.1980). GAG is used to measure the loss of proteoglycan (PG) from tissues into synovial fluids. COMP is a component of load bearing tissues, predominantly in articular cartilage, but it is also present in tendon, ligaments and menisci. COMP concentration in SF may be useful as a prognostic marker of rheumatoid arthritis in people and OA (Marti et al. 1999). COMP levels have also been shown to increase in tendon sheath SF with tendon injury and sepsis (Smith and Heinegård 2000). HA levels have been shown to decrease in horses with OA (Fuller et al. 2001). Metalloproteinases have a major impact on degradation of many cartilage components. Clegg et al. (1998) showed that potential MMP-2 & MMP-9 monomer enzyme activities were significantly elevated in both septic and aseptic joint disease SFs in comparison with fluids from normal joints. MMP-2 and MMP-9 are also useful as biochemical markers for temporomandibular joint disorders (Tanaka et al. 2001).

Broken foot/pastern axes were recorded in 75% of horses suffering from navicular disease, while 45% also *exhibited* mediolateral foot imbalance (Wright 1993a). In cases with joint effusion and a decreased joint synovial space, as is often seen in OA

(Takahashi et al. 1999), imbalance of the hoof *may* result in an increase in the intra-articular pressure in the DIP joint as well as uneven load distribution and thus contribute to damage to articular cartilage. The increasing range of horseshoes and hoofpads makes it important to be able to evaluate their influence on performance and stress in the locomotor apparatus.

The purpose of this study was to investigate biochemical changes in SF, NA, NF and in the DDFT in navicular disease, and to establish if synovial fluid from the DIP joint could be used diagnostically to assess alterations in the synovial fluid of the navicular bursa. We also wanted to test the hypothesis that intra-articular pressure in the DIP joint is significantly higher in legs loaded with heel up (HU), low heel (TU), lateral side up (LU) and medial side up (MU) imbalance compared to the balanced position.

6. REVIEW OF LITERATURE

6.1 Anatomy of the navicular area

The middle phalanx articulates with the combined surfaces of the distal sesamoid (or navicular) bone and the distal phalanx to form the DIP joint. The articular surface of the navicular bone is covered with hyaline cartilage and articulates with the distal extremity of the middle phalanx and the palmar portion of the distal phalanx (Hickman 1964). The palmar surface of the bone is covered with fibrocartilage, and the navicular bursa separates it from the DDFT, which runs over it before inserting onto the distal phalanx. The navicular bone is held in place by two sets of ligaments. The collateral ligaments extend most of the way and are elastic and originate proximal to the medial and lateral depression on the dorso-distal aspect of the middle phalanx and insert onto the medial and lateral extremities of the proximal border of the navicular bone (Sissons and Grossman1975). The relatively less extensible DSIL attaches the distal border of the navicular bone to the flexor surface of the distal phalanx (Parker 1973). Motion in the DIP joint is generally confined to extension and flexion, although some rotation and mediolateral movement is also seen, especially when the horse is moving on uneven ground (Chateau et al. 2002). Due to relative extensibility of the ligaments holding the navicular bone in place, it is usually considered to move in conjunction with the distal phalanx rather than the middle phalanx (Figure 1).

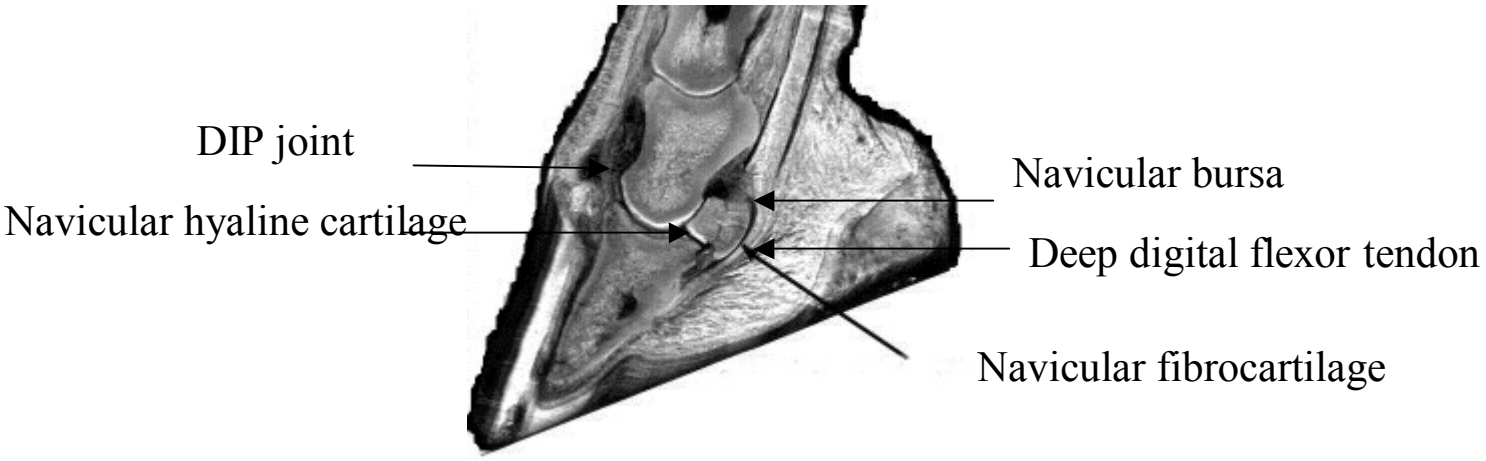

Figure 1. *Sagittal section of a foot*
DIP = distal interphalangeal

6.2 Communication between the navicular bursa and the DIP joint

Direct communication between the DIP joint and navicular bursa is rare, but indirect communication, i.e., filtration between these two structures, is more common. Keegan et al. (1996) showed that mepivacaine hydrochloride deposited into the DIP joint anaesthetizes pain arising from navicular bursal synovia and may decrease pain from the medullary cavity of the navicular bone. Injection of dye into the DIP joint resulted in

diffusion of dye and staining of other structures, including the synovial *coverings* of the collateral ligaments and of the DSIL, and the medullary cavity of the navicular bone. In addition, a blue tinge was observed in the navicular bursa after dye was injected into the DIP joint, suggesting an indirect, and potentially functional, communication between the DIP joint and the navicular bursa (Bowker et al. 1993). It has also been hypothesized that analgesia of the navicular bursa is not selective for the navicular apparatus and that solar pain in some horses can be temporarily abolished or attenuated by analgesia of the navicular bursa (Schumacher et al. 2001). A recent paper reported the incidence of diffusion of mepivacaine between the DIP and the navicular bursa in fresh equine cadaver limbs as being greater than that associated with latex, gelatin dye or contrast media (Gough et al. 2002).

6.3 Analysis of synovial fluid and the navicular bursal fluid

Synovial fluid has been used as an indicator of articular pathology for many years (Van Pelt 1962). In particular, the activities of several enzymes in SF have been determined for evaluating the extent and type of tissue damage in various joint diseases in the horse. SF analysis reflects the status of the synovial membrane, and degenerative disease results in elevated levels of extra cellular matrix (ECM) components in synovial fluids (Lohmander 1997). The relationship between the SF and the pathologies of the synovial membrane and articular cartilage needs to be established. No reports analysing the composition of navicular bursal fluid could be found in current literature.

In synovial fluids of horses with moderate OA, COMP is increased in some breeds but decreased in others (Skioldebrand et al. 2001). This difference between breeds may

reflect different load characteristics and differences in the release of macromolecules between osteoarthritic and normal joints. Misumi et al. (2001) found decreased COMP levels in horses with aseptic arthritis and suggested that COMP degradation in synovial fluids from advanced joint disease may be due to MMP gelatinolytic activity. Levels of synovial fluid GAG reflect cartilage destruction in arthritis, and may be useful for monitoring disease progression in the equine species. High levels of GAG have been found in SF from horses with OA, traumatic arthritis and osteochondrosis (Alwan et al. 1991). HA concentration can be used as a diagnostic marker for chronic traumatic arthritis (Tulamo et al. 1996). HA is responsible for the viscoelastic properties of SF (Laurent and Frasier 1986). It is also used as one of the most common pharmacotherapeutic agents for the treatment of equine joint problems (Howard and McIlwraith 1996). Potential MMP-2 and MMP-9 monomer enzyme activities are significantly elevated in both septic and aseptic joint disease synovial fluids in comparison with fluids from normal joints. These enzymes could therefore be important in the degradation of articular cartilage in joint disease (Clegg et al. 1998).

6.4 Analyses of cartilage and tendon matrix

Although COMP was thought to be a specific molecule in cartilage, and is so named, it is not a highly tissue-specific molecule. Increased levels of COMP in both synovial fluid and serum samples of patients with various joint diseases may be derived not only from cartilage but also from ligaments and tendons (Muller et al. 1998). In tendons, COMP is synthesized in response to, and is necessary for the tendon to resist, load (Smith et al. 1997). In articular cartilage, loading may influence equine COMP distribution (Murray

et al. 2001). COMP serum levels show a strong correlation with the presence and severity of radiographic changes seen in OA.

Proteoglycan degradation is central to the development of OA. Lysosomal enzymes from chondrocytes, synoviocytes or leucocytes may degrade proteoglycans (Spiers et al. 1994). Matrix degradation occurs either by direct damage or by degrading enzymes released into synovial fluid. In human OA, GAG and HA concentrations in SF are decreased (Belcher et al. 1997). Histological and histochemical investigations of the fibrocartilage of horses with navicular disease have shown alterations similar to those described in the hyaline articular cartilage of osteoarthritic joints, leading at least one reseracher to regard navicular disease and OA as analogous processes (Svalastoga and Smith 1983).

A significant difference is present in the concentration of GAG in SF of different joints (Fuller et al. 1996). Load-bearing areas of articular cartilage commonly contain more matrix proteoglycan than non-loaded areas (Slowman and Brandt 1986). Proteoglycan content generally rises with a physiological increase in exercise (Kiviranta et al. 1987).

Cartilage destruction in OA is associated with increased levels of several matrix metalloproteinases, including the gelatinases MMP-2 and MMP-9. While increases in some MMPs may be destructive, up-regulation of others may result from increases in normal tissue turnover (Thompson et al. 2001). Significant increases of MMP-9 monomer and dimer are found in synovial fluids of joints with severe cartilage alterations (Jouglin et al. 2000). Normal equine articular tissues, maintained in short-term tissue culture, produce MMP-2 zymogen alone, while similar tissues obtained from

a variety of pathological conditions produce both zymogen and active MMP-2, as well as MMP-9 monomer and dimer (Clegg and Carter 1999).

The water content of healthy adult equine hyaline cartilage is approximately 70%, and up to 80% in neonates (Todhunter 1996). Hydration values for fibrocartilage vary according to site and function but are generally lower than those of hyaline cartilage. The water content of the canine meniscus is 65% (Stephan et al. 1998). Tendon tissue contains about two-thirds water (Gelberman et al. 1988). Water content has been found to decrease in ageing rabbit tendon (Ippolito et al. 1980). In early OA, water content increases probably due to damage to the collagen network, causing disruption and loss of some matrix proteoglycans (Venn and Maroudas 1977). In contrast, water content has been demonstrated to progressively decrease in damaged fibrocartilagous intervertebral discs (Lipson and Muir 1981). Weaver (2001) noticed that DDFT contained less water than navicular fibro- or hyaline cartilage.

6. 5 Navicular disease

Although the term navicular disease has been in common usage for over 150 years it still defies accurate definition (Wright and Douglas 1993). Historically navicular disease has been used as a term to describe horses with radiological changes of the navicular bone. The term navicular syndrome was introduced probably misleadingly, to describe horses with palmar foot pain, but without radiological changes. It is likely that proportion of those horses had palmar soft tissue injuries and no pathological changes of the navicular bone. Navicular disease should be defined as a disease which results in a chronic, progressive forelimb lameness that is usually bilateral and fits a specific set of

diagnostic criteria (Stashak 2002). At least one of the structures in the palmar aspect of the foot, including the navicular bone, DIP joint, NB, DDFT, collateral ligaments of the navicular bone or DSIL is affected. Various theories have been put forth on the development of navicular disease, but in general these are either biomechanical or vascular in nature. A biomechanical cause has been favoured recently because vascular studies altering the blood supply failed to reproduce the disease (Rijkenhuisen et al. 1989). Navicular disease is estimated to be responsible for one-third of all chronic forelimb lameness in horses (Colles 1982). It remains one of the most controversial and common causes of intermittent lameness in middle-aged horses. A statement made over a century ago still remains valid today: "In no lameness are errors of diagnosis as common as in navicular disease" (Möller 1895).

6. 5. 1 Aetiology

Adams (1969) described navicular disease as a condition, which begins with bursitis of the navicular bursa, between the flexor tendon and the navicular bone, and ultimately leads to degenerative and erosive lesions of fibrocartilage. Pool et al. (1989) proposed a biochemical scheme for navicular disease. He presented histological evidence of bone remodelling in the flexor cortex and adjacent spongiosa, and concluded that the degenerative changes involving the bone are initiated and promoted by exercise and sustained forces of compression exerted against the distal half of the flexor surface. Repetitive abnormal flexion of the distal interphalangeal joint during strenuous locomotor activities may lead to unusual biochemical stresses on associated structures. O'Brien et al. (1975) concluded from pathological, histological and scanning electron

microscopic studies that the changes identified are an attempt by the navicular bone to accommodate the mechanical stress. Doige and Hoffer (1983), MacGregor (1984) and Turner et al. (1986) proposed that the pathological changes in navicular disease are secondary to mechanical damage or increased functional activity of the subchondral bone plate of the flexor surface in response to impact loading or altered mechanical stresses. Horses have also been suggested to respond to pain in the navicular region by contracting the deep digital flexor muscle to unload the heels. This increases the compressive load on the navicular bone, which may cause remodelling and, in some horses, damage to the overlying flexor cartilage, which is then identified as navicular disease (McGuigan and Wilson 2001).

Navicular disease has also been postulated to be an ischaemic disorder resulting from arteriosclerosis and thrombosis in branches of the navicular and digital arteries (Colles and Hickman 1977). However, other investigators have failed to identufy any evidence of thrombosis.

6.5.2 Clinical signs and diagnosis

Navicular disease is generally accepted to be a chronic progressive condition which affects the navicular bone, navicular bursa and the adjacent surface of the deep flexor tendon. It typically affects horses between 6 and 12 years of age. Both front feet are generally involved, but lameness is usually more marked on one side. Turning in the direction of the lame limb exacerbates the lameness in 95% of horses (Wright 1993a). Factors such as faulty conformation, hoof imbalance, improper or irregular shoeing, and exercise on hard surfaces are believed to predispose to the disease (Pool et al. 1989;

Turner 1989; Stashak 1998). Navicular disease is more prevalent in Quarterhorses, Thoroughbreds and Standardbreds than in other breeds in USA (Lowe 1974). Breed-related incidence varies with geographic locations but navicular disease is relatively uncommon in ponies and Arabians. One study in warmbloods found an element of heredity probably related to conformation (Bos et al.1986). Many of the papers characterizing navicular disease describe specific gait changes as being indicative of the condition: in particular, a shortened cranial phase of the stride and toe landing first (Amin et al. 1986, Mac Gregor 1986, Stashak 2002, Wright 1993a).

A thorough systematic examination of the hoof is essential for the clinical diagnosis of navicular disease. There may be evidence of pain when pressure is applied with hoof testers to the overlying hoof and sole (Stashak 2002). However, negative response to hoof testers cannot exclude navicular disease from the list of differential diagnoses and horses with other conditions such as an abscess or bruising of the sole may also show a positive response to the hoof testers. DIP joint extension test may also exacerbate the lameness. A palmar digital nerve block (PDN) results in improvement of the horse's gait. However, this block is not specific for navicular region pain. It has a high predictive value, its ability to detect only navicular disease is low (Turner 1989). Most horses also respond to local analgesia of the distal interphalangeal joint and the navicular bursa (Dyson and Kidd 1993). In one study, DIP joint anaesthesia improved lameness in 92% of the horses diagnosed with navicular disease (Wright 1993a).

Diagnosis of navicular disease is usually based on clinical signs, results of nerve/joint blocks. Radiography is used to support the clinical diagnosis. A minimum of three radiographic projections should be obtained (lateromedial, palmaroproximal-

palmarodistal oblique, dorsal60^{o} proximal-palmarodistal oblique). For comprehensive evaluation of the area desensitised by PDN blocks more radiographic views are needed. Radiographic changes associated with navicular disease include increased number and/or size of distal border synovial invaginations, flattening and/or thinning of the flexor cartilage, proximal border remodelling, medullary trabecular disruption and medullary sclerosis, but differences of opinion exist regarding the radiographic abnormalities and their significance (Wright 1993b). Although radiography is considered an important diagnostic tool, it is effective and sensitive in detecting only advanced pathological changes.

Scintigraphy can be a valuable aid in diagnosing navicular disease in horses, especially when radiographic findings do not support clinical findings (Trout et al. 1991). Scintigraphy identifies early alterations in bone metabolism, making it unnecessary for clinicians to rely on radiographic changes but false positive results can occur. Increased uptake of technetium is not synonymous with either pain or disease, although increased focal uptake in the centre of the bone usually indicates disease (Dyson, personal communication). In future, magnetic resonance imaging (MRI) may prove to be useful in diagnosing navicular disease (Dyson et al. 2003).

Navicular bursography, where a 1:1 ratio of a contrast material and a local anesthetic is injected into the navicular bursa, identifies pathology in the flexor cortex of the navicular bone 60% more often than traditional radiography according to Turner (1996).

6.5.3 Treatment of navicular disease

Various treatment regimens have been recommended for navicular disease. These have consisted of variable periods of rest, corrective shoeing, corticosteroid agents injected into the navicular bursa, non-steroidal anti-inflammatory medications administered parenterally, vasoactive agents and surgery (Ackerman 1977; Colles 1982). Rest is important to allow time for healing to begin and to allow the horse to acclimatize to corrective trimming and shoeing. Generally, rest for three weeks, followed by controlled exercise, is recommended (Stashak 2002). Corrective shoeing is the basis for treatment for navicular disease and is discussed in section 5.8.

The most commonly used drug type for pain alleviation in the treatment of navicular disease is a non-steroidal anti-inflammatory drug (NSAID), in particular, phenylbutazone. NSAIDs are often administered at the beginning of corrective farriery treatment to allow the horse to adjust to the new foot shape and shoes without pain. After a period of rest, controlled exercise is recommended (Kirker-Head 1993). NSAIDs can also be used to manage pain over a longer time period, and many pleasure horses are treated with a "maintenance dose" of phenylbutazone to allow pain-free, light exercise.

Injection of corticosteroids into the DIP joint and the navicular bursa is advised when corrective farriery and NSAIDs are not successful in relieving lameness (Verschooten et al. 1991). Injection into the DIP joint or the navicular bursa is anticipated to have the same effect as analgesia of these structures but lasts for 3 to 12 weeks (Stashak 1998). Hyaluronan can be injected either intrasynovially, alone or together with corticosteroids, or intravenously, although has no proven efficacy. Polysulphated glycosaminoglycans (PSGAGs) can be used intramuscularly or intrasynovially for the treatment of navicular

disease. In one study, intramuscular PSGAGs improved lameness in horses with navicular disease compared with a control group given saline, but follow-up time was only 24 days (Crisman et al. 1993).

Although a vascular pathogenesis of navicular disease has not been validated rheological agents remain popular for treatment. Warfarin is believed to prevent thrombosis in the distal navicular arteries and enhance blood flow to the navicular bone (Colles 1979). Isoxsuprine hydrochloride increases blood flow to the distal portions of the limbs (Rose et al. 1983). Pentoxifylline and propentofylline are synthetic xanthene derivatives used to treat navicular disease. They alter the physical characteristics of the blood, increasing erythrocyte flexibility, and thus, easing blood flow through the capillaries (Kirker-Head 1993).

Recently, a biphosphonate has been studied in clinical trials for its ability to treat navicular disease. Biphosphonates are bone metabolism regulators that inhibit bone resorption, and results have been promising at a dose of 1mg/kg of tiludronate in horses with clinical signs for 6 months or less (Denoix et al. 2002).

Surgical attempts to treat navicular disease include palmar digital neurectomy, desmotomy of the collateral ligament of the navicular bone (navicular suspensory desmotomy) and desmotomy of the accessory ligament of the DDFT (Stashak 1998). Neurectomy desensitizes the palmar third to one half of the foot, thereby removing pain from the navicular region. The outcome of successful surgery is similar to the improvement of lameness seen after a palmar digital nerve block. The rationale behind performing a navicular suspensory desmotomy is that the strain in the navicular suspensory ligament is high at the end of stance and may be a source of pain (Wright

1986). This is supported by the load on these structures being increased by a broken back hoof/pastern axis. Desmotomy of the accessory ligament of DDFT has been performed in selected cases where it has not been possible to correct a broken back hoof/pastern axis. Sectioning that ligament is believed to reduce the force in the DDFT, and hence, the compressive force to the navicular bone. It also helps re-align the hoof/pastern axis (Turner 1992).

As in most diseases, the earlier navicular disease is treated, the more likely treatment will be successful. Often horses with navicular disease remain undiagnosed until the disease has advanced to the stage where irreversible problems exist inside the foot or the conformation and balance of the hoof is irrecoverable (Leach 1993).

6.6 Influences of loading

The stresses generated during joint loading are among the most significant factors affecting articular cartilage integrity. Reduced joint loading has been shown to lead to a decreased PG content of articular cartilage (Kiviranta et al. 1987), with a parallel decrease in HA concentration (Haapala et al. 1996). Weight bearing is an essential component of loading in the maintenance of articular cartilage structure and function. In dogs, the PG content of articular cartilage increased in a limb contralateral to a casted leg in response to increased weight bearing (Tammi et al. 1983), and in rabbit knee cartilage, early osteoarthritic changes developed due to elevated weight-bearing (Paukkonen et al. 1986). However, lifelong increased weight bearing in dogs with normal joints did not cause any alterations in the histological structure and mechanical properties of articular cartilage (Newton et al. 1997).

Horses with navicular disease are thought to alter their gait in response to pain in the heel region (Wright 1993a; McGuigan and Wilson 2001). Corrective shoeing may also alter the angle and alignment of the hoof, and thus, have an influence on the way the horse loads its feet (Willemen et al. 1999).

6.7 Joint pressure

Increased intra-articular pressure causes pain in man (Goddard and Gosling 1988). Horses with synovitis/capsulitis and OA of the MCP joint have increased intra-articular pressures (Strand et al. 1998). Slight increases in joint pressure in the static joint have been associated with damage to articular cartilage and release of MMPs. Effusion and changes in joint orientation may also compromise the blood supply to the bone (Arnoldi et al. 1980). When the SF volume increases in a joint, both loading and movement result in further pressure rises, and therefore, increased risks of damage (Vegter 1987). These changes in volume and pressure during weight bearing and movement are presumably the result of compression of the joint capsule by overlying ligaments and tendons, leading to changes in joint configuration, and hence, capsule tension.

6.8 Imbalance of the hoof and corrective shoeing

Foot balance is the establishment of correct anatomical relationships in the distal limb and is essential for the forces in the foot and the distal limb to remain within physiological limits (Wright and Douglas 1993). In a balanced foot, the line drawn through the axis of the proximal, middle and distal phalanges should be straight. Various abnormalities of the hoof are associated with navicular disease. Heels may be low or

collapsed. In one study, broken foot/pastern axes were recorded in 75% of horses suffering from navicular disease, while 45% also exhibited mediolateral foot imbalance (Wright 1993b). Broken hoof/pastern axis is also often seen in breeds used for racing. Some trainers still believe that low heel and long toe conformation increase stride length and speed. Horses with small upright feet such as Quarterhorses are also prone to development of navicular disease. The large ratio of body weight to foot size may be an important factor.

Of navicular disease cases, 80-90% had one foot narrower than its counterpart (McGregor 1984) but this may be seen associated in any case of lameness and is by no means specific to navicular disease. A broken back hoof pastern axis leads to extension of the DIP joint, with greater forces acting on the palmar region of the foot due to increased strain in the DDFT, and possibly, increased concussion in the palmar structures at impact (McGuigan 2001) (Figures 2 and 3).

Figure 2. Diagrammatic drawing from the lateral side of the equine digit showing hoof-pastern axes on a normal, broken back and broken forward digit.

Figure 3. Diagrammatic drawing of mediolateral imbalance of the foot. The hoof is said to be in balance when an imaginary line through the coronet is parallel to the ground surface and perpendicular to a line that bisects the limb axis when viewed from front.

The clinical literature suggests that horses with painful conditions, such as laminitis (Hood 1999), OA of the intertarsal and tarsometatarsal joints (bone spavin) (Boswell et al. 2000) and navicular disease (Wright 1993a; Stashak 2002), will alter their gait to unload the tissues involved. Some horses may be predisposed to navicular disease as a result of an inherent abnormal gait pattern (Bos 1986).

Corrective farriery aims to restore foot balance and reduce the forces acting on the palmar region of the foot. This usually involves shortening the toe and giving support to the heels in order to reduce the strain, and hence, the force in the DDFT. A variety of different shoeing techniques are recommended, but the two most frequently used are heel wedges and egg-bar shoes (Figures 4 and 5).

Figure 4. Picture of the foot with heel wedge (notice that the branches of the shoe are much too short)

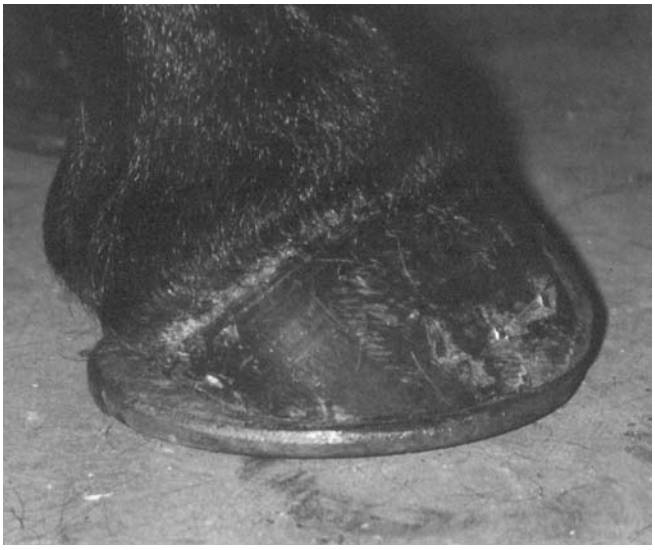

Figure 5. Picture of an egg-bar shoe

Heel wedges raise the heels, restoring the straight hoof pastern axis (if the hoof pastern axis is abnormal) and reducing the extension of the DIP joint. However, use of heel wedges may discourage development of a better-shaped heel in the long run. The egg-bar shoe is oval, extends more palmarly in relation to the foot than the conventional shoe and increases the weight-bearing surface by 30-40% (Moyer and Andersson 1975). The most commonly described techniques for treating navicular disease have been variations of a rolled toe, raised heels, pads (Greeley 1970; Adams 1974) and egg-bar shoes (Ostblom et al. 1984). Corrective farriery should aim to alter the conformation of the hoof to reduce the forces acting on the navicular region. Turner (1986) indicated in his study of shoeing methods that correction of pre-existing hoof problems to align and balance the hoof properly and allow for hoof expansion may allow the horse to function adequately despite the disease. Another study showed that when heel wedges are applied the maximal force on the navicular bone is reduced compared with flat shoes in normal horses (Willemen et al. 1999). However, heel wedges can increase the forces acting on the heel region and may lead to further heel collapse (Ostblom et al. 1982). Raising the heel may also lead to a greater risk of superficial digital flexor tendon damage (Riemersma et al. 1996).

7. AIMS OF THE STUDY

The main objectives of this study were:

1. To evaluate the reproducibility of five different injection techniques of the navicular bursa and to assess which technique is consistently the most successful (I).

2. To determine the normal constituents of navicular bursal fluid and to compare these findings with synovial fluid from the DIP joint and the MCP joint. GAG, COMP, HA, MMP-2, MMP-9 and protein levels were analysed (II).

3. To clarify whether the parameters measured in Study II could be used as biomarkers of navicular disease. Synovial fluids were collected from the DIP joint and the navicular bursa of horses suffering from navicular disease and healthy control horses (III).

4. To assess DNA, GAG, COMP and MMP levels in NA, NF and DDFT from both healthy horses and horses suffering from navicular disease. The same parameters were also measured in collateral cartilage of the hoof and the meniscus of the stifle (IV).

5. To evaluate how imbalance of the hoof affects intra-articular pressure of the DIP joint and to establish the distribution of the joint volume change as a result of imbalance (V).

8. MATERIALS AND METHODS

8.1 Horses

One hundred and twenty-five distal forelimbs were collected from an abattoir for Study I. A range of horse breeds and sizes was used.

Samples for Study II and control samples for Studies III and IV were collected from horses euthanized for reasons other than lameness at the Royal Veterinary College, London. These horses had no history of lameness in their forelimbs. Macroscopic examination was performed and radiographs (see 7.3.2) were taken of all legs used as controls. Synovial fluids were collected before opening the joints.

Horses with navicular disease came from the Royal Veterinary College, London (III, IV), the Faculty of Veterinary Medicine, Helsinki (III), and an abattoir near Bristol (IV). A history was obtained either at the time of the clinical examination carried out at the clinic (III, IV) or via a questionnaire which was sent to the owner of the horse (IV).

Horses were diagnosed as suffering from navicular disease if they had been lame and had shown a positive response to a palmar digital nerve block (PDN). All feet were radiographed either at the time of the clinical examination or after euthanasia. Diagnostic procedures also included scintigraphic examination and the DIP-joint block (5-7 mls of local anaesthetic solution was used) or, in some cases, navicular bursal block (2-3 mls of local anaesthetic used), as outlined in Table 1. Clinical signs, chronic lameness and response to PDN block without radiological signs were the sole criteria of diagnosing navicular disease for one horse in study III and two horses in sudy IV. All

other horses without radiological evidence of navicular disease had *focal* increased uptake of technetium in their navicular bones in scintigraphic examination.

Table 1

Diagnostic tests performed on horses with navicular disease.

	III	IV
Number of horses	23	18
Mean age	10.6 (SE 0.7)	10.8 (SE 0.5)
Bilaterally lame	18/23	15/18
Radiological changes	12/23	10/18
Scintigraphic examination	10/23	8/18
Positive NB block	8/23	7/18
Positive DIP block	12/23	8/18

SE = standard error
NB= navicular bursa
DIP= distal interphalangeal joint

Sixty forelimbs without any macroscopic pathological signs were used in Study V.

No differences between the sexes were observed in these experiments; therefore, the data were not segregated on the basis of sex.

8.2 Handling of samples

8.2.1 Synovial and intrabursal fluid (II, III)

Synovial fluids were collected into test tubes, centrifuged for 10 minutes at 10 000 g and stored in aliquots at –70° C until assayed.

8.2.2 Cartilage and tendon samples (IV)

Full thickness cartilage samples were harvested from the entire articular surface of the NA and NF. DDFT samples were collected from the area opposite to the navicular bone. Additional fibrocartilage samples were collected as follows: the proximal part of the collateral cartilage was obtained from the medial side of the hoof as well as medial meniscus; collateral cartilage and meniscus samples were only collected from healthy middle-aged horses (5-15 years). Samples were assessed macroscopically and stored at -20º C until analysed. Tissue wet weights were determined before freezing.

8.3 Procedures

8.3.1 Injection techniques (I, II, III)

The SF from the DIP joint and the MCP joint was collected in a routine manner (Stashak 1987). A 90-mm, 19-gauge spinal needle (Yale spinal) was used for all injections of the navicular bursa. The distal palmar approach to the 'navicular position' was used to obtain fluid from the navicular bursa in Studies II and III.

Study I, the following five different techniques were used:

1. Distal palmar approach, parallel with the coronary band (Scrutchfield 1977; Stashak 1987; Turner 1989). The needle was inserted midway between the heel bulbs, immediately proximal to the coronary band, and advanced dorsally in the sagittal plane of the limb, parallel with the coronary band, until significant resistance was encountered.

2. Distal palmar approach, parallel with the sole (van Kruiningen 1963; Wheat and Jones 1981; Worthmann 1982; Dyson and Kidd 1993; Grant 1996). The needle was inserted in the same manner as the first approach but parallel with the solar surface of the foot, i.e. horizontally.

3. Proximal palmar approach (Bishop 1960). The needle was inserted into the hollow of the pastern and advanced dorsally and distally in the sagittal plane of the foot, at an angle of 30^{o} to the horizontal, until significant resistance was encountered.

4 .Lateral approach (van Kruiningen 1963; Diez and Wiesner 1984; Turner 1989; Grant 1996). The needle was inserted just proximal to the lateral cartilage of the third phalanx, between the lateropalmar border of the second phalanx and the lateral border of the deep digital flexor tendon, and advanced distally at an angle of 45º to the horizontal in the frontal plane of the limb, until significant resistance was encountered.

5. Distal palmar approach to the 'navicular position. (Verschooten et al. 1991). The navicular position was defined as a point on the lateral hoof wall, 1 cm distal to the coronary band, and halfway between the most dorsal and most palmar aspect of the coronary band. The needle was inserted midway between the heel bulbs, proximal to the coronary band, and advanced in the sagittal plane towards the point bisecting the sagittal plane and the long axis of the navicular bone, until significant resistance was

encountered. The long axis of the navicular bone was assumed to be the connecting line between the 'navicular position' points on the lateral and medial hoof wall.

For techniques 1- 4, the limb was clamped at the level of the proximal metacarpal region just distal to the carpus and pastern, such that the solar surface of the foot was horizontal, to mimic a weight-bearing stance. For technique 5, the limb was clamped at the level of the proximal metacarpal region and the foot was supported in a Hickman block, with the MCP joint and the DIP joint flexed, to mimic a non-weight-bearing position.

8.3.2 Radiography (I, II, IV, V)

Lateromedial radiographs were taken in Study I. In Studies II and III, limbs were radiographed (lateromedial, dorsal60^{o}proximal-palmarodistal oblique, palmar45^{o}-50^{o}proximal-palmarodistal oblique views) to assess lesions characteristic of navicular disease such as remodelling of the proximal and distal borders, enlarged synovial *invaginations* and flexor cortex changes. In Study V, each leg was radiographed (standard lateromedial and dorsal60^{o}proximal-palmarodistal views) to see where the casting material had set (silicone is visible on radiographs).

8.3.3 Papain digestion (II, III, IV)

For the DNA and GAG analysis, all samples were papain-digested. Samples were incubated in 0.25 mg/ml of papain, 0.1 M sodium acetate pH 6.0, 5 mM EDTA, 5 mM cysteine HCL for 2-3 h at 60^{o} C.

8.3.4 Tissue extraction (IV)

Cartilage and tendon were finely diced and extracted twice with 4.0 mol/l guanidine hydrochloride (GnHCl) in 0.05 mol/l sodium acetate buffer, pH 5.8, containing proteinase inhibitors [pepstatin (1.0 µg/ml), 1,10-phenanthroline (1.0 mmol/l), iodoacetic acid (1.0 mmol/l), phenylmethylsulphonyl-fluoride (1.0 mmol/l)] at 4º C for 24 h (Platt and Bayliss 1994).

8.3.5 Determination of DNA (IV)

Cellularity of the cartilages and tendon was determined by assessing the total DNA content of the tissue following papain digestion. DNA was quantified using the bisbenzimidazole (Hoechst 33258) fluorescent dye technique (Kim et al. 1988). The fluorescence of aliquots of the digests was evaluated by spectrofluorimetry: emission and excitation wavelengths of 600 and 365 nm, respectively, were used. Standard curves were constructed using solutions of highly polymerized calf thymus DNA of known concentration.

8.3.6 COMP concentration (II, III, IV)

SF samples were analysed without the GnHCl extraction, necessary for analysis of cartilage and tendon samples. GnHCl extracts of cartilage and tendon were precipitated twice with 95% ethanol, 50 mM sodium acetate (-20º C, overnight), centrifuged at 8 000 g for 30 min and freeze-dried. Pellets were resuspended with sample buffer and the quantity of COMP in the supernatant was determined using a heterologous inhibition ELISA (Smith et al. 1997). Polyclonal antibody raised against equine COMP (Smith et

al. 1997) was used as the primary antibody, and swine anti-rabbit immunoglobulin conjugated to horse-radish peroxidase was used as the secondary antibody. P-nitrophenyl phosphate was used as a substrate and colour intensity was measured at 405 nm with a Labsystem Multiscan microplate reader. All samples were analysed in triplicate, and the mean value was used for calculations.

8.3.7 GAG concentration (II, III, IV)

Total GAG content of tissues and SFs were determined by spectrophotometric assay using dimethylmethylene blue (DMMB) dye (Farndale et al. 1986). Absorbance was measured at 600 nm using a microplate reader.

8.3.8 HA concentration (II, III, IV)

HA was analysed by a colorimetric method (Kuttsch and Schleich 1989). Hyaluronan reacts with the dye 1-ethyl-2-[3-(1-ethylnaphtho-[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl] naphtho -[1,2-d]thiazolium bromide ("stains-all") to form a complex with an absorbance maximum at 640 nm. Samples were diluted with distilled water when needed. The basic assay method was adapted for use in 96-well microtitre trays and measured using a microplate reader.

8.3.9 Determination of matrix metalloproteinases (II, III, IV)

MMP-2 and MMP-9 activity was assayed by gelatin zymography (Sepper et al. 1994). Gelatin was polymerized in 11% polyacrylamide gels with SF and in 8% polyacrylamide gels with cartilage and tendon samples. Synovial fluids were diluted 1:20 with TNC and

mixed with sample buffer in a ratio 1:3. Ten microlitres (µl) of sample mixture was loaded into each lane. Three milligrams of tissue was mixed with 100 µl of sample buffer. Five to fifteen microlitres of that mixture was loaded into each lane. Polymorphonuclear neutrophils, separated from equine blood, were used as an internal standard in each gel (Raulo and Maisi 1998). The relative activity of the bands was assessed using computer-assisted image analysis of the gels (Biorad).

8.3. Total protein concentration (II, III)

Total protein (g/l) was determined using the Biuret method (Doumas et al. 1981). Absorbance was measured at 540 nm, and the method was adapted for use with a microplate reader.

8.3.11 Loading apparatus (V)

Limbs were sectioned through the distal humerus, at a level perpendicular to the long axis of the limb, at mid-stance orientation of the olecranon 3-5 cm above the elbow joint, and a 13 mm- diameter hole was drilled vertically down through the elbow joint into the radius. The cadaver limbs were mounted onto a hydraulic loading jig by means of a 130 mm-long, 12 mm-diameter pin attached to a hydraulic ram, which provided a 50 mm-diameter flat loading surface. This pin locked the elbow joint to prevent any change in joint angle during loading but was not rigidly fixed to the ram to allow hinging.

The hoof of the limb was positioned on a footplate. A shear beam force transducer placed under the footplate recorded axial limb force. The signal from the force

transducer was amplified via a strain gauge amplifier and logged at 100 Hz via software (LabView). The amplified output from the force transducer was displayed on a voltmeter so limb force could be controlled during loading cycles.

8.3.12 Measuring the intra-articular pressure (V)

After mounting the leg onto the jig, a 20 G needle was inserted through the dorsal articular pouch into the DIP joint. Intra-articular pressure was measured by connecting the needle to a fluid-filled cannula, and a three-way tap was connected to a solid state pressure transducer. The output from the pressure transducer was also logged via LabView. Each limb was loaded to 1000 N, which equals the limb vertical force generated during stance by a 450-kg horse, and to 2000 N, the peak force experienced when the horse is walking (Merkens et al. 1985) (Figure 6).

Limb unloaded Limb loaded

Figure 6. Picture of the loading jig showing limb in a loaded and an unloaded position.

The hoof was first positioned in its normal "balanced" weight-bearing position, and the pressure was measured. Using a 5° angle plastic wedge, the hoof angle was then altered to heel up (HU), toe up (TU), lateral side up (LU) and medial side up (MU) imbalance. Intra-articular synovial pressure was measured with the legs loaded at 1000 N and 2000 N in each position.

With eight legs, the intra-articular pressure was measured in all five orientations at 1000 and 2000 N before injecting contrast material into the joint. With twelve legs, 5 ml of contrast material was injected into the joint before the measurements were made.

8.3.12 Silicone casting

Twenty-eight forelimbs were used. In the first group of eight legs, 15 ml of silicone rubber casting material was injected into the joint. In the second group, 20 legs were used and 5 ml of silicone casting material was injected into the joint. Immediately after injecting the silicone, the leg was loaded to 3000 N in the balanced, HU, TU or LU position. Legs were left loaded for 3 h for the silicone to cure.

8.4 Statistical Analysis

Data were analysed using the Cochran-Q, Fischer's Exact *post hoc* and the Kruskal-Wallis tests (I). The Pearson association test was used to assess the correlations between measurements. Mean differences were compared using a non-paired t-test (II, III).

Comparison of means between different groups was calculated using the one-way ANOVA test (IV, V).

9. RESULTS

9.1 Injection techniques (I)

The distal palmar approach (No. 5) was clearly the best and easiest technique to use. The position of the approach to the navicular bone was highly predictable, as a point 1 cm distal to the coronary band and halfway between the most dorsal and most palmar aspects of the coronary band.

9.2 Different control tissues (II, III, IV)

Synovial fluid samples collected from the NB and the DIP joint were different from those collected from the MCP joint in control horses. All of parameters measured from SF correlated significantly between the NB and the DIP joint. COMP, GAG, HA and MMP-2 levels were very similar in fluid from both of these areas.

COMP and GAG values were significantly higher in NA than in other tissues ($p<0.05$). DDFT had the highest MMP-2 content.

GAG content decreased with age in both NA and NF ($p<0.05$). COMP content increased with age in the NA and the DDFT ($p<0.05$). In the NF, the trend was the same, but the difference was not statistically significant. Interestingly, the only significant age-related change in MMP-2 activity was a peak in tendons from middle-aged horses ($p<0.05$). No measurable MMP-9 activity was present in young horses, whereas approximately 50% of old horses had MMP-9 activity in DDFT.

9.3 Navicular hyaline and fibrocartilage and deep digital flexor tendon from horses with navicular disease

Correlation between the NB and the DIP joint composition in individual horses with navicular disease was not as clear as with SF studies in control horses. Only COMP values positively correlated in individual horses. However, as a group, most changes in disease were observed in both compartments. Biochemical changes were also observed with navicular disease in NA, even when no macroscopic changes in this cartilage were noted.

COMP concentrations were very similar in all synovial structures measured (II, III), and COMP was used to standardize other measured parameters. However, in diseased horses, COMP content was significantly increased in NA.

GAG loss in disease was evident in both SF and tissue studies in horses who had lesions in NF. GAG content was also lower in control horses both in NA and NF if they had lesions in NF (IV).

HA concentration and the HA/COMP ratio were higher in synovial fluid from the DIP joint of horses with navicular disease. A difference exists in HA levels of control horses between Studies I and II. No clear explanation for this was found; the difference might be due to the colorimetric assay used. All samples for each study were analysed at the same time to preclude a difference in HA levels interfering with results.

MMP-9 was present more often in SF and tissues of diseased horses than in control groups. MMP-9 and MMP-2 activity levels were higher in SF of diseased horses, and the MMP-2 activity level in NA and DDFT was increased in the tissue study. In DDFT, the activity of MMP-9 was also increased in horses with navicular disease.

Results of biochemical studies (II, III, IV) for middle-age horses (3-14 years) old are presented in Table 2.

Table 2a. Biochemical study results. Synovial fluid and tissue results from healthy horses.

Study			**II**		**III**				
Samples	**SF**		**SF**		**TISSUE**				
	NB	DIP	NB	DIP	NA	NF	T	LC	M
Mean age	13.3 (1.2)		7.8 (0.6)		8.8 (0.6)			10.1 (0.9)	9.9 (1.2)
DNA	-	-	-	-	0.23 (0.03)	0.3 (0.05)	0.33 (0.05)	0.21 (0.02)	0.46 (0.1)
COMP	43.5 (6.3)	58 (12.3)	45 (9)	44 (8)	2.7 (0.3)	1.5 (0.2)	1.7 (0.4)	1.5 (0.4)	2.5 (0.8)
GAG	145 (20.9)	166 (19.2)	119 (13)	164 (26)	52.2 (3.3)	43.5 (3.1)	26.3 (2)	42.2 (4.0)	34.8 (5.5)
HA	392 (27.9)	376 (28.6)	668 (96)	677 (99)	-	-	-	-	-
MMP-2	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	0.4 (0.1)	3.1 (0.9)	5.1 (0.9)	21.7 (5.2)	1.5 (0.3)	1.8 (0.2)
MMP-9	0.4 (0.08)	0.5 (0.08)	0.3 (0.1)	0.4 (0.04)	3.1 (1.2)	2.8 (1.1)	4.1 (1.1)	-	-
Protein	12.9 (1.9)	16.3 (2.1)	13 (2)	13 (2)	-	-	-	-	-
Water%	-	-	-	-	72.5 (0.5)	71.2 (1.2)	61.1 (1.6)	67 (1.9)	61.3 (1.2)

DNA, COMP, GAG and HA in synovial fluids are displayed as μg/ml, and in tissue as μg/mg of wet-weight tissue. MMP-2 and MMP-9 are relative amounts. Protein levels are shown as g/l.
COMP is cartilage oligomeric matrix protein. GAG is glycosaminoglycan. HA is hyaluronan. MMP is matrix metalloproteinases.
NB is navicular bursa. NF is navicular fibrocartilage. T is deep digital flexor tendon. DIP is distal interphalangeal joint.

Table 2b. Biochemical study results. Synovial fluid and tissue results from diseased horses.

Study	II		III		
Samples	**SF**		**TISSUE**		
	NB	DIP	NA	NF	T
Mean age	10.6 (0.7)		10.8 (0.5)		
DNA	-	-	0.18 (0.02)	0.25 (0.01)	0.33 (0.04)
COMP	37 (8)	38 (5)	4.6 (1.0)	2.0 (0.7)	3.1 (0.7)
GAG	110 (19)	105 (10)	40.5 (2.7)	33.8 (2.1)	28.0 (5.0)
HA	690 (100)	986 (117)	-	-	-
MMP-2	1.2 (0.2)	0.7 (0.1)	9.3 (2.2)	8.5 (2.5)	34.7 (5.1)
MMP-9	1.2 (0.4)	0.5 (0.04)	6.9 (2.1)	3.1 (1.2)	25.6 (12.5)
Protein g/l	20 (2)	21 (3)	-	-	-
Water %	-	-	70.3 (1.7)	67.1 (1.9)	63.9 (0.9)

DNA, COMP, GAG and HA in synovial fluids are displayed as µg/ml, and in tissue as µg/mg of wet-weight tissue. MMP-2 and MMP-9 are relative amounts. Protein levels are shown as g/l.COMP is cartilage oligomeric matrix protein. GAG is glycosaminoglycan. HA is hyaluronan. MMP is matrix metalloproteinases.NB is navicular bursa. NF is navicular fibrocartilage. T is deep digital flexor tendon. DIP is distal interphalangeal joint.

9.4 Pressure and contact area

Raising the heels increased intra-articular pressure significantly in the DIP joint, while lowering the heels decreased the pressure compared with the balanced position ($p<0.05$). Mediolateral imbalance did not have a significant effect on the intra-articular pressure. Any deviation from the balanced, normal hoof positioning reduced the contact area of articular cartilage within the DIP joint, as seen in Figure 7.

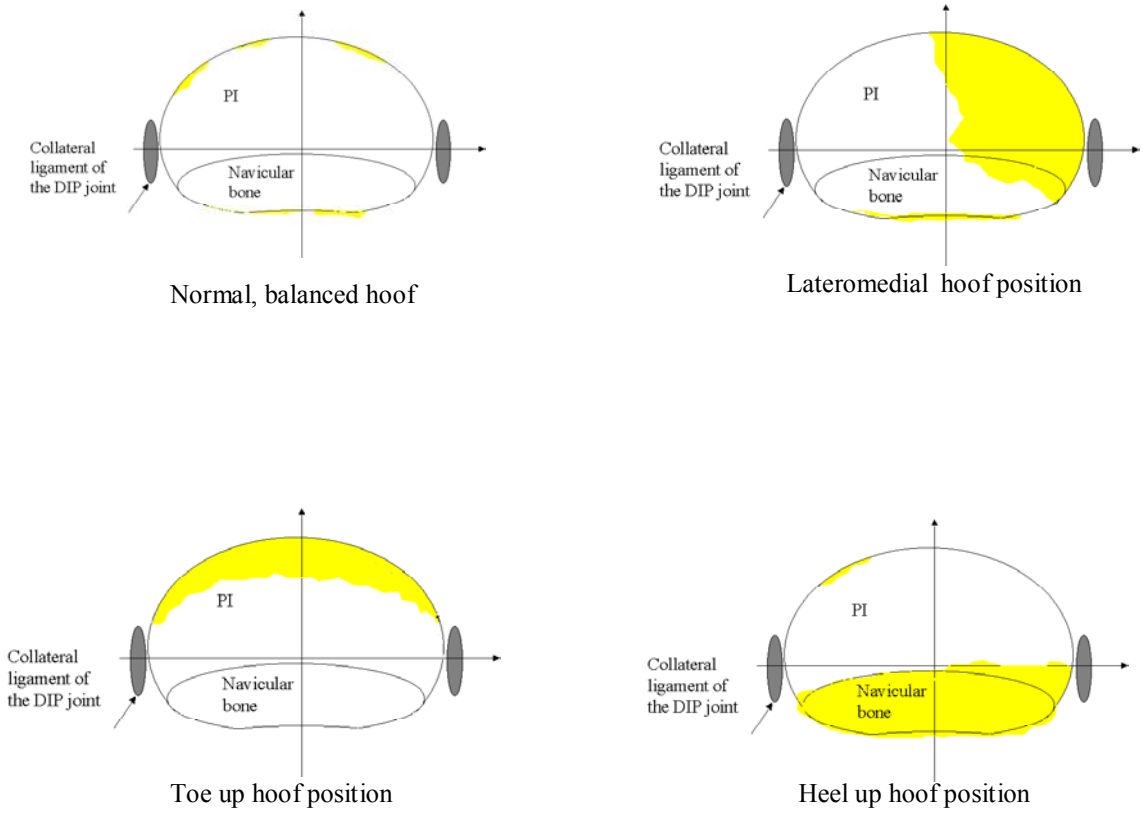

Figure 7. Diagram of the silicone in DIP joint showing reduced contact area in imbalanced feet.

10. DISCUSSION

Numerous techniques for injection of the NB have been described, but there is little conformity between these descriptions. The use of the "navicular position", defined as a point on the lateral hoof wall 1 cm distal to the coronary band and halfway between the most dorsal and most palmar aspects of the coronary band, proved to be a highly predictable landmark for enabling injection. It allowed a high degree of accuracy and reliability of needle placement, irrespective of foot conformation. This was also the best technique to obtain navicular bursal fluid, probably because the DDFT was not pressing against the navicular fibrocartilage in this non-weight-bearing method and was simple to use. We checked needle placement with radiographs. Because the position of the needle is very easy to predict with some practice, in a clinical situation, radiographs may not always be needed. If good markers for identifying navicular disease and staging its progress were found, a reliable injection technique would also allow follow-up samples to be collected in the yard.

Navicular disease is frequently difficult to diagnose. Moreover, interpretation of the results of regional and intra-articular anaesthesia in the horse is often not straightforward (Dyson and Kidd 1993). Analgesia of the PDN is thought to localize pain to the heel region of the foot. However, horses with lameness due to solar toe pain have also been shown to also become sound after analgesia of the PDN (Schumacher et al. 2001). Interpretation of joint blocks is dependent on a number of variables including the volume used, the time after the block at which the horse is evaluated and the

circumstances e. g. straight lines versus circles, under which the horse is evaluated (Dyson 1994). These were standardised as far as possible in studies III and IV. Horses may have pain in their navicular region without any radiological findings, and vice versa; horses without any signs of lameness may have radiological findings commonly associated with navicular disease. Thus diagnosis based on radiographic changes alone is not valid. Scintigraphy may be a valuable aid in diagnosing navicular disease (Turner 1996) but false positive results can occur. Ultrasonographic examination of the foot is limited to the sagittal midline, and artefacts may confuse interpretation (Sage and Turner 2000). Computed tomography (CT) and MRI can be used for more accurate assessment of both bone and soft tissue of the foot. However, they require general anaesthesia and are not widely available (Ruohoniemi and Tervahartiala 1999; Dyson 2002). In this study horses with no radiological changes were included in the navicular disease group. Apart from three horses (one in study III and two in study IV) all these horses had increased focal uptake of technetium in their navicular bones (three horses mentioned did not have scintigraphic examination). Trout et al. (1991) demonstrated the superior sensitivity of nuclear scintigraphy compared with radiography in the diagnosis of navicular disease. It is possible that some horses in the navicular disease group may have been misdiagnosed and were in fact suffering from some other cause of palmar foot pain and interpretation of results must be viewed in the light of the broad definition of navicular disease employed. However, all these horses had been lame for more than 6 months and had clinical signs compatible with navicular disease. Difficulties in diagnosing navicular disease and different opinions of its definition create a clear need

for more sensitive methods both to enable earlier detection of the disease and to learn more about its aetiology

Synovial fluid from the DIP joint and the navicular bursa has the potential to be used to diagnose navicular disease. However, using synovial fluid for analyses has its problems. In diseases of synovial structures, synovial fluid volume often increases as a result of inflammatory infiltrate, possibly diluting the measured parameters. The actual volume of synovial fluid in a joint has been calculated experimentally (Ekman et al. 1981), but it is not usually known in a clinical setting. Furthermore, wide variation in values often exists for different parameters within a joint (Viitanen et al. 2000). Duration of clinical signs and stage of the disease varies and is not always known. History of clinical signs and horses' workload is usually collected from the horse owners and it is therefore not always reliable, especially when horses are used as general riding horses and not for competitive purposes. We should be trying to define much more precisely what is navicular disease and what are the other conditions that mimic the disease, but are primary soft tissue injuries. In broad definition of navicular disease used in this study disease affects many different structures and that probably results in even more variation within samples. Workload and recent work history may also have an effect on synovial fluid values but there is very little information available as how exercise affects SF composition. Further work is also required to validate the repeatability of results of marker concentrations if horses are kept under the same management regime. This is crucial if a technique is to be clinically viable. Ideally, in this study we would have liked to analyse more samples as well as have access to more detailed work histories of the

horses. Further work is also needed to relate the results to stage of the disease and to learn more how the concentrations measured alter in different exercise regimes. The relatively high number of veterinarians performing lameness workups may have resulted in some variability of the inclusion criteria for navicular disease. Ideally the study should be repeated in an experimental model of navicular disease, but to date such a model does not exist. Despite the limitations of the synovial fluid studies, we showed that changes in the concentrations of GAG, HA, MMP-2 and MMP-9 in synovial fluid obtained from the navicular bursa and the DIP joint can be detected in navicular disease with a profile different from that seen in control horses. Because variation in all the measured parameters was great, none alone would be sufficient to diagnose the disease, even the changes found were statistically significant. Local synovial inflammation and clinical subgrouping may influence these measures, supporting different pathogeneses within the navicular disease group. Aetiology of navicular disease is multifactorial and different tissue types are affected. In one study, 18 horses with palmar foot pain without radiological abnormalities were examined using MRI; seven had primary lesions of the DDFT, seven primary lesions of the navicular bone, two osteoarthritis of the DIP joint and two several lesions associated with the insertion of the DDFT on the distal phalanx, the DSIL, the navicular bone and the DIP joint (Dyson et al. 2003). Thus, careful patient characterization is required in synovial fluid studies. Limitations of synovial fluid studies include lack of knowledge of work history, lack of knowledge on the influence on work on the concentrations measured and the absence of repeatability studies. SF measurements may be particularly useful to assess disease progression and response to

treatment in individual horses. Using more than one marker would likely yield more reliable results.

Our findings showed that it is not possible to compare the levels of SF parameters to reference values without knowing the reference values for the particular joint. In addition, the variability between horses within a joint is large. NB and DIP joint values correlated well in healthy horses, indicating communication between these two structures. The MCP joint was very different from the NB and the DIP joint. Its SF values were closer to reference values for normal equine SF (Fuller et al. 1996, Palmer et al. 1995). By contrast, values from healthy DIP joints and the NB were similar to those obtained from joints with traumatic arthritis (Palmer et al. 1995, Tulamo et al. 1996). However, navicular bones and DIP- joints from healthy horses were radiographed, and gross postmortem examination revealed no signs of joint disease. Fuller et al. (1996) have reported large changes in SF between different joints in horses, so our finding *of* large differences between joints is not unusual. Load-bearing areas of articular cartilage also commonly contain more matrix proteoglycan than non-loaded areas (Slowman and Brandt 1986), and this may be reflected in higher concentrations of proteoglycans in SF from joints under high loads compared to less loaded joints.

In diseased horses, there was a correlation between the SF concentrations of the different markers in the NB and the DIP joint but this was not as clear as in healthy horses. While both the DIP joint and NB fluid changes showed similar trends in disease, the levels of different markers in individual horses varied. These results, despite suggesting functional similarity between the NB and the DIP joint, show that these two

compartments do not communicate freely in diseased horses. The SF changes could be a reflection of a parallel disease process rather than molecular equilibration between adjacent synovial structures. In addition, our tissue study supported DIP joint involvement in navicular disease. Although the levels of measured parameters varied, probably due to different tissue types in the navicular region, most of the changes observed in the navicular fibrocartilage and the DDFT in horses with navicular disease were present in the navicular hyaline cartilage, as well. This indicates that changes seen in the synovial fluid of the DIP joint in horses with navicular disease originate at least partly from the navicular hyaline cartilage. Navicular hyaline cartilage is either affected to a lesser degree or is better able to adapt to the factors causing fibrocartilage damage, because even in severe cases of navicular disease no gross pathology develops on this side (Weaver 2001). Further studies are needed to assess the exact level and the type of communication between the navicular bursa and the DIP joint.

Clinically, the viscosity of fluid from the DIP joint and the navicular bursa appears to vary; however these fluids were generally less viscous than that of the MCP joint. Since viscosity was not specifically evaluated in this study, this is merely a subjective observation.

COMP was used to standardize SF measurements because COMP levels were very similar in different joints and in both control and diseased horses. However, COMP content was significantly higher in navicular hyaline cartilage of horses with navicular disease and was also elevated in their navicular fibrocartilage and DDFT, although the

difference was not statistically significant. COMP content has been shown to increase in both navicular hyaline and fibrocartilage of horses with navicular disease (Weaver 2001). Interestingly, in our study hyaline cartilage responded to adverse conditions by increasing COMP production to a greater degree than fibrocartilage, although macroscopically no degenerative changes in any of the hyaline samples were seen. This may indicate that hyaline chondrocytes are more efficient at synthesizing COMP than fibrochondrocytes. The increase in COMP is possibly also indicative of increased fibrillogenesis as part of a repair response. An increase in subchondral navicular bone density occurs in reaction to increased loading. This is also seen in navicular disease as a consequence of factors such as predisposing conformation (Pool et al. 1989), with COMP being produced in response to increased loading (Smith et al. 1997). It is also possible that hyaline cartilage is able to react to altered loading or weight bearing to a greater extent than fibrocartilage. However, it is difficult to explain why COMP levels remained unchanged in synovial fluids. Perhaps an increase in amount of COMP was masked by the diluting effect of increased SF volume in diseased horses.

COMP content increased with age in NA, NF and DDFT. All of these areas are under substantial loading, which may explain the increase in COMP content (Cherdchutham et al. 1999). COMP has been identified in tendons and is thought to function by resisting loading in tendon (Smith et al. 1997). COMP is also thought to have a role in fibrillogenesis. Accumulation may be the result of a response to loading, inducing collagen turnover over a period of time.

As with SF concentrations in the NB and the DIP joint, the mean GAG value obtained for NA was significantly higher than values for other hyaline cartilages (Vachon et al. 1990). However, as different techniques are used to measure GAG, the value of directly comparing absolute results between the studies is questionable. In our study, GAG content decreased significantly with age in NA and NF. However, no decrease occurred in GAG content of DDFT with age. Analysis of the aggregated aggrecan population has indicated an increase in heterogenity with age, suggesting a gradual cleavage in the chondroitin sulphate-bearing region of aggregan, resulting in a reduced GAG content in cartilage (Ratcliffe et al. 1988)

One of the substantial features of OA is loss of proteoglycan, and therefore GAG, from articular cartilage (Nimmi and Deshmukh 1973). The GAG content decreased in diseased horses which had lesions only on their fibrocartilage in both the NA and in the NF itself. The GAG loss was also evident in SF from horses with navicular disease as compared with the control group synovial fluids. Little information is available on proteoglycan content in other fibrocartilages as they degenerate. However, human meniscal fibrocartilage degeneration coincides with an increase in both proteoglycan synthesis and content (Ghosh and Taylor 1987). Interestingly, GAG content was also significantly decreased in sound horses with lesions on NF. In these horses, GAG was decreased in the NA and NF. These results indicate that GAG loss seems to be more associated with loss of tissue than clinical signs of the navicular disease process or pain. With these control horses, which had lesions in NF but no clinical signs of lameness or pain, it is difficult to predict whether clinical lameness would have developed later. This further suggests that the current clinical criteria for determining navicular disease are not

precise and some horses may remain undiagnosed. It also suggests that navicular disease may have a pain-free phase or that the pain threshold of horses varies.

HA is commonly used to treat equine articular disorders. Clinically achievable concentrations of HA and polysulphated GAG inhibit prostaglandinE_2 (PGE2) synthesis by cultured equine synoviocytes. This anti-inflammatory action may be a mechanism through which these agents exert anti-arthritic effects (Frean and Lees 2000). Both HA and the HA/COMP ratio were increased in SF of horses with navicular disease. Increased concentrations of HA may reflect localized processes secondary to synovial proliferation. Increased serum levels of HA in inflammatory joint diseases (Bjork et al. 1989; Rayan et al. 1998) and in synovial fluid in people with degenerative joint disease (Praest et al. 1997) are well documented. In contrast to our study and a number of OA studies in man and dog (Manicourt et al. 1995; Thonar et al. 1995; Sharif et al. 1996), decreased concentrations of HA have been reported in traumatic arthritis joints in horses (Tulamo et al. 1996).

MMP-2 activity was significantly higher in all tendon tissues than in other tissue types. This could indicate a greater ECM turnover in tendon tissues due to higher forces found in the DDFT in the navicular area. That young horses contained no MMP-9, while in old horses MMP-9 was present in 50% of samples, could indicate age-related accumulation of microtrauma in tissues. MMP-2 activity was increased in SF and NA, and DDFT in horses with navicular disease. This is in accordance with findings that MMP-2 is abundant in naturally occurring joint disease as well as in healthy normal joints, and its

concentration is significantly higher in diseased joints than in normal joints (Trumble et al. 2001). While increases in some MMPs may be destructive, up-regulation of others may result from increases in normal tissue turnover (Thompson et al. 2001). Much less immunostaining for MMPs and cytokines is observed in the deep zone of all OA specimens where the cartilage matrix and chondrocyte morphologies appear normal. Full-thickness normal cartilage specimens show virtually no immunostaining for MMPs or cytokines (Tetlow et al. 2001). MMP-9 activity level was increased in SFs and tendons of horses with navicular disease. MMP-9 was also present more often in synovial fluids and tendons of horses with navicular disease than in healthy horses. MMP-2 and MMP-9 are able to degrade aggrecan core protein in a similar fashion to other MMPs (Fosang et al. 1992; Lark et al. 1997). MMP-cleaved aggrecan has been identified in synovial fluid of human patients with arthritis (Fosang et al. 1996). MMP-9 expression has also been demonstrated in human OA cartilage (Tsuchiya et al. 1997). In the horse, MMP-2 and MMP-9 levels have been found to be elevated in synovial fluid in articular disease (Clegg et al. 1997a).

Most lesions in navicular disease occur near the sagittal ridge of the navicular bone (Pool et al. 1989, Weaver 2001). Had the samples had been collected only from the affected areas, differences between the groups might have been more obvious. However, with this smaller amount of tissue, it would not have been possible to complete all the analyses in this study with the techniques used. Significant changes in GAG composition of OA cartilage from distinct regions have been recorded in humans (Burkhardt et al. 1995). Cartilage matrix characteristics also vary within the same joint

(Ghosh et al. 1975, Adams 1981; Richardson and Clark 1991). A technical consideration regarding collection of samples from NF is that the tissue is very thin, and harvesting sufficient tissue to use in analyses, while simultaneously ensuring that no subchondral bone is included, is problematic.

The development of navicular disease is complex and multifactorial, with controversy arising over the relative importance of the various contributory factors. Corrective shoeing is the basis for treatment for navicular disease. Most of the principles of farriery have been derived from practical experience and traditional skills of individual farriers (Greeley 1970). Therefore, opinions or policies on horse shoeing vary greatly between farriers and members of the veterinary profession. Also, because of specific desires of some owners and trainers many horses are shod improperly in an attempt to fit what is described as desirable effect. There is also a breed variation in hoof shapes and sizes, large Quarter horse having small feet as an example. In one study, broken back hoof axis was recorded in 75% of horses suffering from navicular disease (Wright 1993a). As early as 1829, Turner was convinced that a long toe is a "significant mischief!". Interestingly, our study showed that raising the heels will significantly increase the intra-articular pressure of the DIP joint. The increased intra-articular pressure may directly cause pain and can also decrease blood flow to the synovium and intra-articular ligaments (Hardy et al. 1996). These, in turn, may lead to hypoxia, acidosis and decreased glucose concentration within the synovial fluid, as detected in human patients with inflammatory joint disease (James et al. 1990), contributing to the destructive processes within a joint. In healthy joints, the synovial pressure is usually

subatmospheric (Levick 1983). In the present study, raising the heels lead to intra-articular pressure in the DIP joint above 0 kPa in four out of five horses, even without any artificial fluid distension of the joint. However, another study concluded that shoes with heel wedges reduce the force on the navicular bone as a result of a decreased moment of force at the DIP joint in combination with a decreased angle between the DDFT tendon distally and the navicular bone proximally (Willemen et al. 1999). Therefore, in horses suffering from navicular disease, heel wedges could be anticipated to have a beneficial effect on the pressure exerted on the navicular bone. If heels are raised in these horses, it should be done carefully to avoid masking the beneficial effects to the navicular side by an increase in pressure on the DIP joint side, causing pain and possible progression of the disease on that side.

With the normal "balanced" foot orientation, there was good contact between the joint surfaces, and most of the silicone was squeezed away from the entire articular surface during loading. This would indicate that in this position the largest and most even contact area was present between the proximal and distal joint surfaces of the DIP, and thus, the least pressure per unit area. Eggbar shoes are commonly used to treat horses suffering from navicular disease. These shoes have no effect on the force on the navicular bone in sound horses (Willemen et al. 1999), but a significant unloading effect is observed in some horses with navicular disease (McGuigan et al., in preparation). The mechanism of action is unclear but may relate to redistribution of load over a larger heel or reinforcement/coupling of the flexible palmar regions of the foot.

Wedging and any alterations in foot orientation resulted in silicone remaining on the articular surface of the wedged side. This indicates uneven load distribution across a

joint and localized higher pressures on the contact cartilage surfaces with any deviation from the "balanced" foot position. *In vivo*, this would lead to greater localized "wear and tear" on the joint surface and possibly a greater tendency towards OA. The results support the view that the balanced foot, where a line drawn through the axes of the proximal, middle and distal phalanges is straight, is ideal.

This study has provided evidence that degeneration of navicular fibrocartilage is an active process which has the potential to be modulated. The DDFT is likely to be involved in the pathogenesis of navicular disease, and horses with navicular disease have changes in their DIP joints, which may contribute to the lameness seen in clinical cases. We have also shown that alterations in the hoof angle have an effect on intra-articular pressure in the DIP joint and on the volume/contact area in the joint. The imbalance of elevated heels may be detrimental for the long-term viability of the DIP joint as well as of the navicular bone itself.

11. CONCLUSIONS

1. The injection technique "distal palmar approach to the navicular position" was found to be the most reliable. With this technique, the navicular bursa was located correctly in 92% of legs on the first attempt.

2. A significant correlation was revealed between the levels of COMP, GAG, HA, MMP-2, MMP-9 and protein in the DIP and NB but no correlation with the MCP, suggesting either free movement of molecules between SF of the DIP joint and NB or a functional linkage between synovial composition in the two structures.

3. We found a reduction in synovial fluid GAG concentration in both the DIP and NB of horses with navicular disease, as well as an increase in HA in the DIP joint and an increase in MMP-2 and MMP-9 in the DIP joint and NB of all diseased horses. However, the correlation of levels of the different molecules measured between the DIP joint and NB, in control horses was not maintained in diseased joints, suggesting that the changes were a reflection of parallel disease processes rather than molecular equilibration between adjacent synovial structures.

4. Horses with navicular disease have increased amounts of COMP in their NA. MMP-2 is increased in the DDFT and NA. The GAG content is lower in NA and NF of diseased joints, the same structures being affected in sound horses with lesions in NF. This supports the involvement of the DIP joint as well as the palmar aspect of the navicular bone and the bursa in navicular disease. Matrix changes occur in NA, NF and DDFT, with potential implications in the pathogenesis of the condition.

5.Changes in foot orientation, which could result from trimming and shoeing, were found to have an effect on intra-articular pressure in the DIP joint and on the

volume/contact area of the joint. The results support the view that a balanced foot is ideal and the imbalance of elevated heels may be detrimental to the long-term viability of the DIP joint.

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